Comment

Toxicity Assessment of Tobacco Products *in Vitro*

Joseph R. Manuppello[1] and Kristie M. Sullivan[2]

[1]People for the Ethical Treatment of Animals, Norfolk, VA, USA; [2]Physicians Committee for Responsible Medicine, Washington, DC, USA

Summary — Driven by new regulatory demands to demonstrate risk reduction, the toxicity assessment of tobacco products increasingly employs innovative *in vitro* methods, including biphasic cell and tissue cultures exposed to whole cigarette smoke at the air–liquid interface, cell transformation assays, and genomic analyses. At the same time, novel tobacco products are increasingly compared to traditional cigarettes. This overview of *in vitro* toxicology studies of tobacco products reported in the last five years provides evidence to support the prioritisation of *in vitro* over *in vivo* methods by industry and their recommendation by regulatory authorities.

Key words: *AIR-100, air–liquid interface, cell transformation, cigarette smoke, cytotoxicity, e-cigarette, inhalation toxicology,* in vitro*, smokeless tobacco.*

Address for correspondence: *Joseph R. Manuppello, c/o People for the Ethical Treatment of Animals, Regulatory Testing Division, 501 Front Street, Norfolk, VA 23510, USA.*
E-mail: *josephm@peta.org*

Introduction

In the USA, the *Family Smoking Prevention and Tobacco Control Act*, which became law on 22 June 2009, gives the Food and Drug Administration (FDA) the authority to regulate the manufacture and marketing of tobacco products. In its guidance for industry, the FDA does not explicitly require that animal studies be conducted in support of applications for the pre-market review of new tobacco products or modified risk tobacco products (MRTPs). However, it does include *in vivo* studies among the types of non-clinical studies that it recommends to address and compare the toxicities of tobacco products. This raises concern that large numbers of animals may be used in studies that are intended to support tobacco product marketing applications. This commentary is adapted from public comments on the FDA's draft guidance for industry on MRTP applications, that were sumitted on behalf of People for the Ethical Treatment of Animals and the Physicians Committee for Responsible Medicine.

The *Tobacco Control Act* directs the FDA to consult with the Institute of Medicine (IOM), a non-governmental organisation founded under the congressional charter of the National Academy of Sciences, on the design and conduct of studies for the assessment of MRTPs. In its report, *Scientific Standards for Studies on Modified Risk Tobacco Products* (1), the IOM quoted a 2009 review by Johnson *et al.* (2) (the pre-print online copy of which is known as 'Wan *et al.*'), in which the authors concluded that *in vitro* tests were not "reliably quantitative to allow valid comparisons of substantially different tobacco products", and that the data provided could not "reliably be extrapolated to infer human cancer risk". However, the authors also observed that most *in vitro* testing had been "limited to a small number of cytotoxicity and genotoxicity assays", with "only isolated studies" comparing one type of tobacco product to another.

In this overview of the many varied and technologically advanced studies that have been reported since 2009, we show that these observations no longer accurately describe the study of tobacco product toxicology *in vitro*, calling into question the inclusion of animal studies among the non-clinical studies needed to support marketing applications.

The accompanying tables summarise recent *in vitro* studies not cited by Johnson *et al.* (2). They are arranged by assay type and whether cigarette smoke or smokeless tobacco was used. The order loosely follows that of the tables presented by Johnson *et al.* (2), to which the reader is referred for further discussion of the endpoints measured, smoke conditions, test substances, and abbreviations. Studies were selected from results returned

by searching the PubMed database for terms such as '*in vitro*' and 'cigarette smoke' or 'smokeless tobacco' from 2009 through to the present.

Air–Liquid Interface Exposure

One technological development that has been employed in many of the recent studies described below is the exposure of cells grown in biphasic culture at the air–liquid interface (ALI). Such exposure systems are the subject of a 2013 review by Thorne and Adamson (3). These authors observe that, while exposure to whole smoke can present technical challenges, it is more physiologically relevant than exposure to conditioned medium, as it captures interactions of both the particulate and vapour phases. Examples of commercially available systems are described in detail, including those from Borgwaldt, Burghart and Vitrocell®; several custom-made systems are also described. The authors note that such systems have been used to assess a variety of airborne chemicals not limited to tobacco smoke. The authors also discuss the emerging field of dosimetry for smoke exposure, which is the subject of a more recent comparison of tobacco smoke particle deposition *in vitro* (3–5). Whole smoke exposure systems allow better control and quantification of the actual exposure concentrations of smoke and particulates at the exposed tissue than is possible with *in vivo* experiments.

Cytotoxicity

Recent studies on the effects of cigarette smoke on cytotoxicity are summarised in Table 1; studies of smokeless tobacco are summarised in Table 2.

Aufderheide *et al.* (6) characterised the CULTEX® Radial Flow System exposure device by exposing bronchial epithelial cells at the ALI to whole smoke from commercial and reference cigarettes, and found a dose-dependent reduction of cell viability. Rach *et al.* (7) reported comparable results with this system, by using bronchial epithelial cells, as well as a decrease in β-tubulin, which was assessed to investigate changes in cytoskeletal structure (8). The authors concluded that the high reproducibility of their results indicates the reliability of the method, and recommended its integration into inhalation studies. Nara *et al.* (9) used this system to expose Chinese hamster ovary cells to the water-insoluble gas/vapour phase (GVP) fraction of cigarette smoke, noting that, for cells grown in submerged culture, this fraction is typically excluded. While the authors found GVP to be less cytotoxic than whole smoke, exposure still resulted in a significant, dose-dependent effect. Similarly, Thorne *et al.* (10) adapted a modified BALB/c 3T3 Neutral Red Uptake test methodology to measure the cytotoxicity of whole smoke and GVP generated by using the Vitrocell VC 10 exposure system; GVP caused significantly less cytotoxicity and variability than whole smoke (10). The authors concluded that these findings demonstrate the importance of testing the entire tobacco smoke aerosol, not just the particulate fraction. The authors observed that this methodology could also be used to assess the toxicities of traditional cigarettes, blend and filter technologies, tobacco smoke fractions, and individual test aerosols.

Li *et al.* (11) used the Vitrocell exposure system to evaluate the effects of test material and smoking regimen on cytotoxicity. Total particulate matter (TPM) or whole smoke from commercial, experimental and reference cigarettes was collected under the smoking machine methods of either the International Organisation for Standardisation (ISO; 35ml puff volume, 2-second puff every 60 seconds) or Health Canada Intensive (HCI; 55ml puff volume, 2-second puff every 30 seconds with complete ventilation block). With both methods, the relative differences in cytotoxicity among the test cigarettes were significantly higher with whole smoke than with TPM. The authors concluded that whole smoke should be the preferred test substance for smoke toxicity, and that intensive smoking methods are less likely to underestimate exposure of smokers. Consistent with these results, Arimilli *et al.* (12) found that whole smoke-conditioned medium (WS-CM) induced higher cytotoxicity than TPM in human monocyte-macrophage cells and human peripheral blood mononuclear cells (PBMCs). In addition, the authors found that combustible tobacco product preparations induced higher cytotoxicity than either smokeless tobacco preparations or nicotine, and that the major PBMC subsets showed differential cytotoxicity when exposed to either WS-CM or TPM, with the greatest cytotoxicity observed in CD4 cells, followed by CD8 cells, monocytes, and natural killer cells.

While most cytotoxicity assays continue to assess plasma membrane permeability, pH gradients, or redox potential, Chen *et al.* (13) described a real-time cellular impedance biosensor-based method for comparing the cytotoxicities of cigarette smoke condensates (CSCs). The authors used the time-dependent IC50 values obtained to determine the maximum toxicity of cigarette smoke and the reversibility of toxic effects, both of which are reportedly difficult to measure by using other assays. In addition, the method features automatic detection, easy operation, and no cell labelling, making it suitable for routine comparisons of the cytotoxicities of CSCs.

In recent years, cytotoxicity has also been assessed in a greater variety of cell types. Richter *et*

Table 1: Cytotoxicity studies on cigarette smoke and electronic cigarette vapour

Assay	Cell type	Product (MRTP); conditions	Substance	Results (reference)
WST-1 (tetrazolium salt reduction)	16HBE14o- (bronchial epithelial cell line)	K3R4F (9.4mg tar), K1R5F (1.67mg tar); ISO	Whole smoke (WS), air–liquid interface (ALI)	No cytotoxic effect was found after exposure to mainstream smoke of one cigarette (K3R4F). By increasing the number of cigarettes, cell viability decreased. Exposure to eight cigarettes induced an almost complete loss of cell viability. The exposure of the cells to the low-tar cigarette revealed no sign of toxicity, if the cells were exposed to the smoke of up to ten cigarettes. Cytotoxic effects were primarily visible after exposure to the smoke of 12 and more cigarettes. (6)
CellTiter-Blue Cell Viability Assay (metabolic activity)	16HBE14o-, Calu-3 (human submucosal gland cell line)	K3R4F; ISO	WS, ALI	An increased number of cigarettes induced a continuous decrease in this biological endpoint to approximately 20% cell (16HBE14o-) viability after eight cigarettes. In contrast to the 16HBE14o-, cell viability values were increased in Calu-3 cells after the exposure to one, two, four and six cigarettes, as compared with the clean air exposed control cultures. The mean cell viability of Calu-3 cells was slightly lower than that observed in the control cultures only after exposure to eight cigarettes. (7)
Neutral Red Uptake	CHO-K1 (Chinese hamster ovary cell line)	2R4F; ISO	Gas vapour phase (GVP), conditioned PBS, ALI	This test atmosphere induced a clear cytotoxic effect, which increased by decreasing the dilution of the extracted gas vapour phase. At a dilution of 0.2L/min for the water-insoluble gas vapour phase, the NR uptake was reduced to 71%, while that for the corresponding water-soluble and insoluble atmosphere was about 29%. (9)
Neutral Red Uptake	BALB/c 3T3 (mouse embryo cell line)	3R4F; ISO	WS, ALI; GVP, ALI	Results from whole smoke exposure across airflows of 1.0–10.0L/min gave a dilution IC50 of 6.02L/min with relative percentage survival ranging from 100–0 percent viability, when compared against the concurrent air control. The GVP data also showed a consistent dose–response between experiments, generating an average dilution IC50 of 3.20L/min over a 184 minute exposure period. (10)
Neutral Red Uptake	CHO	Three experimental cigarettes; ISO, Health Canada Intensive (HCI)	Total particulate matter (TPM), Cambridge method; WS, ALI	Under both ISO and HCI regimens, the relative differences in cytotoxicity among the test cigarettes indicated by the EC50 values in WS were significantly higher than those in TPM. For TPM testing, cytotoxicity was decreased, going from the ISO regimen to the HCI regimen, consistent with the reported reductions of toxicant output on a per unit of TPM basis under the HCI regimen. For WS, cytotoxicity increased for the two lower TPM cigarettes, and decreased for the higher TPM cigarette going from the HCI regimen to the ISO regimen. (11)
7-Aminoactinomycin D (7-AAD; intercalation into double-stranded DNA)	THP1 (human monocyte macrophage cell line); PBMC (human peripheral blood mononuclear cells)	3R4F; ISO	TPM, Cambridge method; WS, conditioned media (CM)	Treatment with TPM and WS-CM at low nicotine units (2–6μg/ml) resulted in significant cell death compared to ST/CAS and nicotine. Cell death in cells exposed to TPM and WS-CM reached 100% at substantially low nicotine units (2–4μg/ml and ~6μg/ml, respectively). Human PBMCs appeared to be more sensitive to WS-CM treatment at lower range compared to exposure to TPM, whereas in HL60 and THP1 cell lines were more sensitive to TPM. (12)
Cellular impedance biosensor (CIB) based method	CHO-K1	Five commercial cigarettes with different tar yields; ISO	Cigarette smoke condensate (CSC), Cambridge method	After a 5-hour exposure, all the concentrations of CSCs induced a gradual decline in the cell survival rate in a dose-dependent manner. With higher concentrations of CSCs, CI could be reduced to zero which means no viable cells adhere to the microelectrodes. With lower concentrations of CSCs, the cells were able to continue growing with a slope similar to that of the control cells in DMSO-only wells during their log phase at approximately 15 hours after addition of CSCs, representing the cell recovery responses to toxic agents. (13)

Results are presented in the original authors' own words, adapted for clarity.

Table 1: continued

Assay	Cell type	Product (MRTP); conditions	Substance	Results (reference)
Multiple cytotoxicity endpoint (MCE; microscopic observation of Giemsa-stained cells)	TK-6 (human lymphoblastoid cell line)	2R4F, commercial (US): 'light' non-menthol (LT), 'light' non-menthol (LTMAS), full-flavour, non-menthol (FF), non-menthol with charcoal filter (CHAR); experimental: 100% reconstituted (REC), 100% flue-cured (BRI), 100% Burley (BUR); ISO	CSC, Cambridge method	With metabolic activation, BRI had the lowest EC50. EC50s for 2R4F and CHAR were similar. EC50s for LT and LTMAS were also similar. The highest measured EC50 was BUR. Condensates FF and REC did not achieve a 50% reduction in cell growth at the tested concentrations. In the test system without metabolic activation, 2R4F had the lowest EC50, followed by CHAR, BRI, and BUR. LT showed an EC50 near the highest concentration tested. LTMAS, FF, and REC did not achieve an EC50 at the tested concentrations. (14)
MTT (tetrazolium salt reduction)	HMVECL (Human microvascular endothelial cells from the lungs), NHBE (normal human bronchial epithelial cells), SAEC (human small airway epithelial cells); IdMOC multiple cell-type co-culture system	2R4F, LT, LTMAS, FF, CHAR, REC, BRI, BUR; ISO	CSC, Cambridge method	In NHBE, excluding BRI, the reduction in viability ranged from 1.5-fold (BUR and CHAR) to 5.6-fold (2R4F). Condensate BRI was the most cytotoxic in NHBE cells. All test concentrations of BRI reduced viability to no more than 49%. The EC50 for BRI was 15μg/ml. The EC50s for 2R4F, LT, LTMAS, FF, and REC ranged from 92 to 132μg/ml. The EC50s for condensates BUR and CHAR were more than 200μg/ml. In SAEC, CSCs produced between a 1.3-fold (REC) and a 17-fold (2R4F) range in reduction in viability. Condensate BRI was the most cytotoxic in SAEC cells with an EC50 of 46μg/ml. Moderate cytotoxicity occurred with 2R4F, LTMAS, and FF (EC50 ranged from 128 to 170μg/ml). The lowest cytotoxicity occurred with condensates LT, REC, BUR, and CHAR (EC50s greater than 200μg/ml). (14)
Neutral Red Uptake	BALB/c 3T3, CHO	2R4F, LT, LTMAS, FF, CHAR, REC, BRI, BUR; ISO	CSC, Cambridge method	Condensate 2R4F was slightly to mildly cytotoxic at 50μg/ml and above. Condensates LT, LTMAS, and REC showed slight and mild cytotoxicity at 100 and 200μg/ml, respectively. Condensate FF was slightly and moderately cytotoxic at 100 and 200 μg/ml, respectively. Condensate BUR showed slight to moderate cytotoxicity at 50μg/ml and above. Condensate CHAR produced mild and moderate cytotoxicity at 100 and 200μg/ml, respectively. Condensate BRI was the most cytotoxic, showing mild to severe cytotoxicity at 50μg/ml and above. Condensates 2R4F, LT, and BUR showed slight and mild cytotoxicity at 100 and 200μg/ml, respectively. Condensate LTMAS and FF were slightly toxic at 50 and 100μg/ml while LTMAS was mildly cytotoxic and FF was moderately cytotoxic at 200μg/ml. The highest test concentrations of REC and CHAR were only mildly cytotoxic. Condensate BRI was mildly cytotoxic at 100μg/ml and severely cytotoxic at 200μg/ml. (14)
Neutral Red Uptake	HMy2.CIR (human B-cell lymphoblastoid cell line)	Commercial (China), no. (mg tar/mg CO/mg nicotine): 1 (15/12/1.3), 2 (8/10/0.7), 3 (5/8/0.6), 4 (3/3/0.3), 5 (15/15/1.3), 6 (15/13/1.3), 7 (7/8/0.7), 8 (8/8/0.8), 9 (12/13/1.0), 10 (14/15/1.3), 11 (15/14/1.3), 12 (15/15/1.3); ISO	CSC, Cambridge method	As compared with DMSO, a significant decrease ($p < 0.05$) in cell viability was observed at the doses of 2.5–12.5 × 10^{-3} cigarette/ml for No. 1, 5, 6 and 9–12 CSCs, at the doses of 7.5–12.5 × 10^{-3} cigarette/ml for No. 7 and 8 CSCs ($p < 0.05$) and at the doses of 10.0–12.5 × 10^{-3} cigarette/ml for No. 2 and 3 CSCs. The cytotoxicity of No. 10 CSC was the highest among 12 CSCs by whatever means, and the range of the cytotoxic potency among 12 CSCs was 9.69-fold, 2.05-fold, and 2.24-fold when the cytotoxic potency was expressed as per 1 × 10^{-3} cigarette, per 1 × 10^{-3} mg tar, and per 1 × 10^{-3} mg nicotine, respectively. (15)

Table 1: continued

Assay	Cell type	Product (MRTP); conditions	Substance	Results (reference)
CCK-8 (tetrazolium salt reduction)	HMy2.CIR	Commercial (China): no. (mg tar/mg CO/mg nicotine) 1 (15/12/1.3), 2 (8/10/0.7), 3 (5/8/0.6), 4 (3/3/0.3), 5 (15/15/1.3), 6 (15/13/1.3), 7 (7/8/0.7), 8 (8/8/0.8), 9 (12/13/1.0), 10 (14/15/1.3), 11 (15/14/1.3), 12 (15/15/1.3); ISO	CSC, Cambridge method	As compared with DMSO, a significant decrease ($p < 0.05$) in cell viability was observed at the doses of $2.5–12.5 \times 10^{-3}$ cigarette/ml for No. 6 and 8–12 CSCs, at the doses of $5.0–12.5 \times 10^{-3}$ cigarette/ml for No. 1, 2, 5 and 7 CSCs, at the doses of $7.5–12.5 \times 10^{-3}$ cigarette/ml for No. 3 CSC and at the doses of $10.0–12.5 \times 10^{-3}$ cigarette/ml for No. 4 CSC. The cytotoxicity of No. 1 CSC and No. 2 CSC was the highest among 12 CSCs when the cytotoxic potency was expressed as per 1×10^{-3} cigarette, per 1×10^{-3} mg tar, and per 1×10^{-3} mg nicotine, respectively, and the range of the cytotoxic potency among 12 CSCs was 6.43-fold, 1.70-fold, and 1.97-fold when the cytotoxic potency was expressed as per 1×10^{-3} cigarette, per 1×10^{-3} mg tar, and per 1×10^{-3} mg nicotine, respectively. (15)
XTT assay (tetrazolium salt reduction)	HUVEC (human umbilical vein endothelial cells)	Unknown	CSC	50 and 100mg/ml CSC reduce the number of viable cells significantly, as compared with the exponential growth of control cells. (16)
Propidium iodide staining (nuclear DNA)	HUVEC	Unknown	CSC	Treatment with 100mg/ml CSC leads to the degradation of nuclear DNA, in contrast to cells treated with 50mg/ml CSC and compared to control cells. (16)
Lactate dehydrogenase (LDH; released through damaged cell membranes)	HUVEC	Unknown	CSC	Incubation with 50mg/ml CSC has no effect on membrane integrity, whereas incubation with 100mg/ml CSC induces a massive release of LDH, which started after 48 hours of incubation. (16)
Sulphurhodamine B binding (estimates number of viable cells)	101A, 101B (oral squamous cell carcinoma cell lines), HGEC (human gingival epithelial cells)	3R4F; ISO	TPM, Cambridge method; WS, CM	With increasing doses of TPM, up to 80% cytotoxicity for 101A and 101B cells was observed while the maximal cytotoxicity for HGECs was close to 60%. Substantial cytotoxic effects were observed following WS-CM treatment; the maximal cytotoxicity of WS-CM was 90%, 80%, and 34% in 101A, 101B, and HGEC cells, respectively. (17)
Neutral Red Uptake	V79 cell line	Experimental cigarettes w/wo novel tobacco blend treatment (BT; MRTP); ISO	TPM, Cambridge method	The IC50s of the TPMs from cigarettes with BT tobacco were not different from those of TPMs from cigarettes without BT tobacco. (18)
Neutral Red Uptake	V79 cell line	Experimental cigarettes with tobacco-substitute sheet (TSS; MRTP), Silk Cut King Size (SCKS) filtered; ISO	TPM, Cambridge method	On a TPM and NFDPM (nicotine-free dry particulate matter) basis, the IC50 values obtained for experimental cigarettes containing TSS were higher than those of matched control cigarettes, although the values were not statistically significantly different for S620 which contained 30% TSS, the lowest level tested. When the IC50 values were calculated on an nicotine- and humectant-free dry particulate matter (NHFDPM) basis, i.e. allowing for the glycerol content of the NFDPM phase, then no statistically significant differences between any of the samples were seen. (19)

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Table 1: continued

Assay	Cell type	Product (MRTP); conditions	Substance	Results (reference)
Neutral Red Uptake	BALB/c 3T3	2R4F, experimental cigarettes with activated charcoal (AC) filter; ISO, Massachusetts Department of Public Health (MDPH)	TPM, Cambridge method; GVP	Compared on a per cigarette basis, the cytotoxic activity of the TPM fraction was slightly lower in the smoke of the AC filtered cigarette than in that of the control cigarette; however, this difference was not statistically significant. For the vapour phase fraction of the AC filtered MS, no EC50 value could be determined due to the lack of measurable cytotoxicity, even though the concentration range tested was extended by three-fold compared to the control cigarette. The significant reduction of the cytotoxic activity of the vapour phase of the AC filtered cigarette smoke correlated with a –92% change of the acrolein concentration. (20)
Neutral Red Uptake	BALB/c 3T3	2R4F; Electrically Heated Cigarette Smoking System (EHCSS, MRTP): EHCSS-K6, EHCSS-K6M, EHCSS-K3, 6mg tar delivery: M6UK (UK), M6J (Japan), 1mg tar delivery: Philip Morris One (PM1), Lark One (Lark1); ISO, human puffing behaviour (HPB)	TPM, Cambridge method; GVP	Cytotoxicity of TPM and GVP from both EHCSS-K6 and EHCSS-K3 was lower than that of M6UK and M6J. Cytotoxicity of TPM from the ECHSS-K6 was comparable to that of PM1, but higher than that of Lark1, while that of the EHCSS-K3 was lower than that of both PM1 and Lark1. Cytotoxicity of GVP from the ECHSS-K6 was higher than that of both PM1 and Lark1, while that of the EHCSS-K3 was lower than that of PM1, but higher than that of Lark1. Cytotoxicity showed a broad response range as a function of smoking intensity for all cigarettes smoked under HPB conditions. (21)
MTT	H9c2 (cardiomyoblast cell line)	Commercial cigarette (0.8mg nicotine, 10mg tar/10mg carbon monoxide); twenty commercially-available electronic cigarette liquids (EC, MRTP); ISO	WS, CM; EC vapour, CM	CS extract was cytotoxic at extract concentrations > 6.25%. Three EC extracts (produced from tobacco leaves) were cytotoxic at 100% and 50% extract concentrations and one ('Cinnamon-Cookies' flavour) was cytotoxic at 100% concentration only. IC50 was > 3 times lower in CS extract compared to the worst-performing EC vapour extract. Vapour produced by the 'base' liquid was not cytotoxic at any extract concentration. Cell survival was not associated with nicotine concentration of EC liquids. (22)
LDH release	HaCaT (keratinocytes), A549	UK research cigarette (12mg tar, 1.1mg nicotine), e-CIG mixtures (balsamic flavours with or without nicotine, Cloudsmoke, Terna Trade; MRTP)	WS, ALI; EC vapour, ALI	Exposure to cigarette smoke caused an early and progressive decrease in cell viability and increased LDH release with a similar trend during the different time points in both cell lines, although keratinocytes seem to be more susceptible to WS induced toxicity after 24 hours. Exposure to EC vapour in which both flavouring substances and nicotine were absent resulted in no change in either LDH release or cell viability over 24 hours. Exposure to EC with flavour caused significant increase of LDH release and a progressive loss of viability in both cell types, although HaCaT cells seem to be more susceptible than A549 cells. Even more dramatically, EC with nicotine caused rapid and marked loss in viability and enhanced LDH release, exhibiting a quantitative and qualitative response superimposable to that of WS exposure. (23)
MTT	Primary human skin fibroblasts	Unknown	CSE	A dose-dependent reduction in the number of viable cells at 12 hours, which reached statistical significance at 1.25% CSE was observed. At the 1.25% concentration of CSE, a 27.2% inhibition of cell viability was observed. (26)

Table 1: continued

Assay	Cell type	Product (MRTP); conditions	Substance	Results (reference)
Trypan Blue dye-exclusion	A549, BEAS-2B (bronchial cell line)	A: non-filter cigarette made in Holland by British American Tobacco (BAT; 10mg tar/8mg CO/0.8 nicotine) B: non-filter cigarette made in Italy by BAT (10/7/0.9) C: filter cigarette made in Italy by BAT (9/8/0.8) D: filter cigarette made in EU by Japan Tobacco (8/9/0.7mg CO/0.8 nicotine); smoke was aspirated into a glove box chamber by a vacuum pump	CSE	The Trypan Blue exclusion test on cells exposed for 30 minutes to CSE did not show a statistically significant reduction in cell viability of A549 cells compared with unexposed control cells, whereas a significant cell viability reduction was observed in BEAS-2B cells after exposure to 5 and 10% CSE from unfiltered B cigarettes. (33)
MTT	A549, BEAS-2B	A, B, C, D (above); smoke was aspirated into a glove box chamber by a vacuum pump	CSE	The MTT assay with BEAS-2B cells exposed to CSE for 24 hours showed a decrease, even if not statistically significant, in cell viability for CSE of unfiltered B cigarettes at 5 and 10%, and a lack of significant effects for the other cigarettes, while in A549 cells, no significant viability changes were observed compared with control cells (33).
LDH release	A549, BEAS-2B	A, B, C, D (above); smoke was aspirated into a glove box chamber by a vacuum pump	CSE	Results of the LDH assay after a 30-minute exposure showed membrane damage, evaluated as a statistically significant increase of LDH release compared with control cells, for B cigarette in A549 cells and for C cigarette in BEAS-2B cells, at 10% CSE. A lack of increase of LDH release compared with control cells was found in A549 cells for the CSE from D cigarettes at all concentrations and for the A cigarette from 2.5 to 10% CSE, whereas in BEAS-2B cells no membrane damage was found for the A cigarette (at 1.25% and 5% CSE) and for the B cigarette (at 5% CSE). LDH results after a 24-hour exposure showed membrane damage for the B cigarette in both cell lines. In particular, in A549 cells a statistically significant increase in LDH release compared with control cells was found at 10% CSE of cigarette A, and at 1.25% and 2.5% CSE of cigarette B. A lack of increase of LDH release compared with the control was found for cigarette D. In BEAS-2B cells, a significant increase of LDH release compared with the control was found only at 10% CSE of cigarette B, and a lack of increase of LDH release was found for the A and C cigarettes. (33)
Clonogenic survival assay	FE1 (cell line derived from MutaMouse lung epithelium)	Brand 1: flue-cured, full-flavour Brand 3: blonde, king size Brand 5: flue-cured, light, king size; ISO	CSC	The results for Brand 1 indicate no reduction in survival at concentrations less than approximately 78μg TPM/ml, and Brands 3 and 5 did not elicit reduced survival at concentrations less than approximately 88 and 80μg TPM/ml, respectively. At 90μg TPM/ml, all brands showed slight cytotoxicity and yielded clonogenic survival values of 85, 95, and 82% of controls for Brands 1, 3 and 5, respectively. Above 90μg TPM/ml, all brands showed substantial cytotoxicity, and yielded survival values that rapidly dropped to below 60% of the control. However, statistical analyses showed a significant drop in clonogenic survival at the highest concentration, relative to the control, only for Brands 1 and 5 ($p < 0.05$, one-sided t-test). Statistical analyses failed to reveal any significant differences in cytotoxicity between the three brands. (34)

Table 1: continued

Assay	Cell type	Product (MRTP); conditions	Substance	Results (reference)
CCK-8	Human peripheral lymphocytes	Commercial cigarette (15mg tar, 1.3mg nicotine)	CSC, Cambridge method	The percentages of viable cells went from 100% to 47.09% with S9 and from 97.18% to 83.54% without S9. The viable cell percentages at the doses of 50–100μg/ml in CSCs with S9 were significantly elevated, as compared with the corresponding DMSO control. Moreover, when the percentages of viable cells at the same doses were compared between CSCs with and without S9, it was found that percentages of viable cells at the doses of 100 and 125μg/ml in CSCs with S9 were significantly higher than those in CSCs without S9. (64)
CCK-8	HMy2.CIR	Cigarette 1 (3mg tar, 0.3mg nicotine, 3mg CO), Cigarette 2 (15/1.3/15); ISO	CSC, Cambridge method	The percentages of viable cells were from 91.04% to 83.84% in the cigarette 1 group, and from 90.2% to 12.28% in the cigarette 2 group. The viable cell percentages at the doses of 10.0–12.5 $\times 10^{-3}$ cigarette/ml in the cigarette 1 group, and at the doses of 5.0–12.5 $\times 10^{-3}$ cigarette/ml in the cigarette 2 group, decreased significantly ($p < 0.01$), as compared with corresponding DMSO controls. Moreover, when the percentages of viable cells were compared between the cigarette 1 group and the cigarette 2 group at the same doses, it was found that percentages of viable cells at the doses of 5.0–12.5 $\times 10^{-3}$ cigarette/ml in the cigarette 2 group were significantly lower than those in the cigarette 1 group ($p < 0.01$). (65)
LDH release	hMVEC (human lung microvascular endothelial cells)	2R4F, 1R4F	CCS (conditioned cigarette smoke; blow-by system used to mimic exhaled smoke exposure)	The relative levels of LDH in the medium containing the cells exposed to 3–4 exposure units (EU), of CCS are significantly higher than those in the medium containing control cells, indicating CCS-induced cell injury. (66)
WST-1	hMVEC	2R4F, 1R4F	CCS	The relative level of WST-1 in the cells exposed to 1 EU of smoke is higher than that in the control cells. This suggests that exposure to low levels of CCS stimulates cell growth. The relative levels of WST-1 in the cells exposed to 2–4 EU of CCS are significantly decreased in a dose-dependent manner, indicating that exposure of hMVEC to high levels of CCS decreases cell viability. (66)
Neutral Red Uptake	BALB/c 3T3	3R4F, M4A, TSS (MRTP), BT (MRTP); ISO	TPM, Cambridge method	The TSS PMs had higher IC50 values than their respective controls in both repeat experiments, indicating lower cytotoxicity. BT1 also differed from its control, but the effect was not reproducible. The IC50 values of PMs from cigarettes with 6mg ISO tar yields, were higher than those of PMs from cigarettes with 1mg tar yields, in two repeat experiments. (67)

al. (14) compared CSCs from commercial, experimental and reference cigarettes in three different cytotoxicity assays with unique and overlapping endpoints (multiple cytotoxicity endpoint [MCE]). Human microvascular endothelial cells from the lungs, normal human bronchial epithelial (NHBE) cells, and human small airway epithelial cells, were cultured in the IdMOC™ multiple cell-type co-culture (MCTCC) system. While exposure to all the CSCs reduced cell viability, there was little correlation between CSCs producing morphological evidence of toxicity and CSCs with the lowest EC50 values (concentration required for 50% cytotoxicity) in the MCE or MCTCC assays; however, CSCs from cigarettes made with flue-cured tobacco were the most cytotoxic across the assays. As described in Table 1, Lou *et al.* (15) found that exposure to CSCs from each of 12 commercial cigarettes reduced the viability of human B-lymphoblastoid cells, while Messner *et al.* (16) reported decreased cell viability and loss of membrane integrity of human umbilical vein endothelial cells (HUVECs) exposed to CSC (16). Gao *et al.* (17) exposed human gingival epithelial cells (HGECs) and oral squamous cell carcinoma cells to whole smoke, TPM and smokeless tobacco extract (STE), and reported greater decreases in cell viability for HGECs, as well as much less cytotoxicity for STE, when the effects of concentrations of extracts were compared over a similar range of nicotine equivalents.

The cytotoxicity of potential MRTPs has also been investigated recently. Combes *et al.* (18) and McAdam *et al.* (19) evaluated the effects of TPM from experimental cigarettes made with either tobacco substitute sheet (TSS) that contains glycerol to dilute toxicants or tobacco that had been blend-treated (BT) to reduce precursors of tobacco-specific *N*-nitrosamines and polyphenols. These treatments were then further combined with adsorbent, activated carbon, or multi-stage filters. The authors reported reduced cytotoxicity for TPM from TSS cigarettes, and concluded that reduced toxicity cigarettes can be developed without introducing additional hazards. Consistent with these results, Gaworski *et al.* (20) found that the reduced yield of vapour phase irritants from activated charcoal filter cigarettes correlated with markedly decreased *in vitro* cytotoxicity and less pronounced morphological changes of the nose and lower respiratory tract *in vivo*. Zenzen *et al.* (21) reported generally lower cytotoxicity for TPM and GVP from Electrically Heated Cigarette Smoking System cigarettes compared to lit-end cigarettes. Similarly, Farsalinos *et al.* (22) evaluated the cytotoxicity of the vapour from 20 commercial electronic cigarettes on cultured myocardial cells, and found that, while some were cytotoxic at the highest concentrations tested, all were significantly less cytotoxic than WS-CM from a commercial lit-end cigarette (22). Finally, Cervellati *et al.* (23) reported that in human keratinocytes and epithelial lung cells, the cytotoxic constituents of electronic-cigarette vapour were limited to the flavouring compound and, to a lesser extent, nicotine. The authors concluded that electronic-cigarette vapour is far less toxic than whole smoke.

Apoptosis

Recent studies on the effects of cigarette smoke on apoptosis are summarised in Table 3; studies of smokeless tobacco are summarised in Table 4.

Richter *et al.* (14), mentioned above, found that, with metabolic activation, the frequencies of apoptotic cells for all the CSCs tested were comparable to solvent controls, while trend tests for full-flavour, burley and charcoal-filtered CSCs showed a positive dose–response relationship for necrotic cells. Without metabolic activation, 'light' CSC showed a positive dose–response relationship for apoptotic and necrotic cells, while full-flavour and reconstituted CSCs showed a positive dose–response relationship for necrotic cells. In the MCE assay with human lymphoblastoid cells, the CSCs that had the highest EC50 values for reduced cell growth also showed a positive dose–response relationship for necrotic cells. Lou *et al.* (15), also mentioned above, found that apoptosis was induced upon exposure to all the CSCs tested.

Kosmider *et al.* (24) investigated the effects of WS-CM exposure on human alveolar epithelial type I-like (ATI-like) cells. By using fluorescent staining and terminal dUTP nick end-labelling, the authors detected the disruption of mitochondrial membrane potential, apoptosis and necrosis. In addition, WS-CM exposure induced translocation of nuclear factor-erythroid 2 related factor 2 (Nrf2), and increased its expression, along with that of haeme oxygenase 1 (HO-1), Hsp70, and Fra1. Further, Nrf2 knockdown sensitised ATI-like cells to WS-CM, while Nrf2 over-expression, *N*-acetylcysteine and trolox protected cells. The authors noted that their results are in agreement with observations that reactive oxygen species (ROS) activate Nrf2, which promotes the expression of HO-1 mRNA (25). They concluded that Nrf2 activation is a major factor in cellular defence of the human alveolar epithelium against cigarette smoke-induced toxicity, and hypothesised that antioxidant agents that modulate Nrf2 would reduce cigarette smoke-related lung injury and the development of emphysema.

Yang *et al.* (26) investigated the effects of WS-CM exposure on the growth, proliferation and senescence of skin fibroblasts (26). Dying cells were stained with senescence-associated β-galactosidase (SA β-gal). The level of intracellular ROS was assessed by the 2′,7′-dichlorfluorescein-

Table 2: Cytotoxicity studies on smokeless tobacco products

Assay	Cell type	Product (MRTP); conditions	Results (reference)
7AAD	THP1, PBMC	2S3, complete artificial saliva (CAS)	Cytotoxic effects of ST/CAS and nicotine were observed at much higher levels of nicotine units (than with cigarette whole smoke and TPM). Detectable cell death was observed in cells treated with nicotine and ST/CAS only at several hundreds of microgrammes of nicotine units. Consistent with published data, maximal cell death with nicotine treatment was observed at > 3.0mg/ml. (12)
Sulphurhodamine B binding	101A, 101B, HGEC	2S3, CAS	ST/CAS showed much less cytotoxicity in 101A, 101B and HGECs when a similar range (compared to cigarette smoke) of nicotine equivalents was tested. Even at much higher doses, up to 3 × dilution of the 10% stock, ST/CAS had only maximal cytotoxicity of 25–30% for 101A and 101B cells, and no measurable effect on HGECs. (17)
Neutral Red Uptake	BALB/c 3T3	2S3, water three commercial Swedish snus (SWS; MRTPs): G, water CPS, water CDM, water experimental (flavouring agent) SWS: CDM2, water, DMSO	The increasing cytotoxicities of the extracts from samples G, CPS and 2S3 were very similar, with the lowest attained survival being approximately 75%. At the highest extract concentration, the mean cell survival for the CPS sample was significantly greater (by about 10%) than those for the G and 2S3 samples. The decline in cell survival in the CDM and CDM2 (water extraction) samples with increasing extract concentration was effectively identical, with zero survival at the highest concentration. Cell survival for the CDM2 sample (DMSO extraction) at the highest concentration was 44%. (35)

Table 3: Apoptosis studies on cigarette smoke

Assay	Cell type	Product (MRTP); conditions	Substance	Results (reference)
Caspase 3 activation	THP1, PBMC	3R4F; ISO	TPM, Cambridge method; WS, CM	The participation of caspase 3 in cell death pathways varied, depending on the treatment. For example, WS-CM treatment appeared to activate caspase 3 to well above the concentrations required to induce cell death, suggesting a potential for non-apoptotic cell death. TPM, on the other hand, profoundly activated caspase 3 in a dose-dependent manner, indicating a significant participation of caspase 3-mediated apoptotic cell death. (12)
MCE	TK-6	2R4F, LT, LTMAS, FF, CHAR REC, BRI, BUR; ISO	CSC, Cambridge method	With metabolic activation, the frequency of apoptotic cells for all condensates was comparable to solvent controls. The frequency of necrotic cells was increased for FF at 200μg/ml, BUR at 150μg/ml and 200μg/ml, and CHAR at 150μg/ml and 200μg/ml. Trend tests for FF, BUR, and CHAR showed a positive dose–response relationship for necrotic cells. In the test system without metabolic activation, only condensate LTMAS produced an increase in the frequency of apoptotic cells at 200μg/ml relative to solvent controls. The frequency of necrotic cells was higher for FF at 150μg/ml and 200μg/ml, and for LTMAS and REC at 200μg/ml. Trend tests for LTMAS showed a positive dose–response relationship for apoptotic and necrotic cells. Trend tests for FF and REC showed a positive dose–response relationship for necrotic cells. (14)
Annexin V, fluorescence-activated cell sorting	HMy2.CIR	Commercial (China), No. (mg tar/mg CO/mg nicotine): 1 (15/12/1.3), 2 (8/10/0.7), 3 (5/8/0.6), 4 (3/3/0.3), 5 (15/15/1.3), 6 (15/13/1.3), 7 (7/8/0.7), 8 (8/8/0.8), 9 (12/13/1.0), 10 (14/15/1.3), 11 (15/14/1.3), 12 (15/15/1.3); ISO	CSC, Cambridge method	All the CSCs could induce cell apoptosis in the present study, but the doses of CSCs at which the significant increased percentages of apoptosis cell appeared were different among the 12 CSCs. As compared with DMSO, the significantly enhanced percentages of apoptotic cells ($p < 0.05$) were observed at the doses of $2.0–5.0 \times 10^{-3}$ cigarette/ml for No. 1, 5, 6, 9, 10 and 12 CSCs, at the doses of $3.0–5.0 \times 10^{-3}$ cigarette/ml for No. 2, 7, 8 and 11 CSCs, at the doses of $4.0–5.0 \times 10^{-3}$ cigarette/ml for No. 3 and 4 CSCs. The cytotoxicity of No. 1 CSC was the highest among the 12 CSCs by whatever means, and the range of the cytotoxic potency among the 12 CSCs was 8.19-fold, 2.29-fold, and 3.05-fold when the cytotoxic potency was expressed as per 1×10^{-3} cigarette, per 1×10^{-3} mg tar, and per 1×10^{-3} mg nicotine, respectively. (15)
AIF (apoptosis-inducing factor), immunofluorescence-based analysis	HUVEC	Unknown	CSC	Incubation with 50mg/ml CSC caused a change in the cellular AIF distribution from a more mitochondrial to a nuclear distribution — clearly visible after 72 hours of CSC incubation. However, immunofluorescence staining of endothelial cells incubated with 100mg/ml CSC showed no apoptosis-like translocation of AIF to the nucleus. (16)
Annexin V	HUVEC	Unknown	CSC	Incubation with CSC led to an increase in the number of apoptotic cells and, to a much lesser extent, to an increase in necrotic cells. (16)
Caspase 3 activation, Western blot	HUVEC	Unknown	CSC	Neither the incubation with 50μg/ml CSC nor 100μg/ml CSC induced the activation of caspase 3 over a time course from 24 to 72 hours. 50μg/ml CSC was not only unable to induce caspase 3 activity but even significantly repressed caspase 3 activity in cell-free extracts. (16)
DHR 123 (dihydro rhodamine) oxidation	HUVEC	Unknown	CSC	After 5 hours of incubation, a significant and massive increase in the production of reactive oxygen species (ROS) with 50mg/ml and 100mg/ml of CSC was observed. (16)
P53 activation, Western blot	HUVEC	Unknown	CSC	Incubation with 50mg/ml CSC induced a temporary increase in P53 expression at 24 hours. However, after 48 and 72 hours of CSC incubation with 50mg/ml, no induction and stabilisation of P53 was detectable. Incubation with higher CSC concentration (100mg/ml) caused no P53 induction. (16)

Table 3: continued

Assay	Cell type	Product (MRTP); conditions	Substance	Results (reference)
Annexin V	101A, 101B, HGEC	3R4F; ISO	TPM, Cambridge method; WS, CM	For 101A and HGEC cells, TPM predominantly led to necrosis, while the effect for 101B cells was almost equally divided between apoptosis and necrosis. Interestingly, WS-CM also did not lead to significant increases in either apoptotic or necrotic cells, suggesting that its cytotoxic response as determined by SRB assays may be mainly due to other mechanisms such as cell cycle arrest and/or inhibition of proliferation. (17)
Caspase 3 activation	101A, 101B, HGEC	3R4F; ISO	TPM, Cambridge method; WS, CM	TPM significantly increased caspase 3 activity in 101A, 101B cells (~3- to 8-fold) and HGECs (~70-fold), although the latter had very low basal and induced levels throughout any of the treatment and control conditions. Thus, caspase 3 activities paralleled the SRB toxicity data for 24-hour TPM treatment, indicating mainly caspase-dependent apoptotic cell death. WS-CM significantly increased caspase 3 activity in HGECs (~5-fold), whereas a similar stimulation was not observed in 101A and 101B tumour cells. In this case, caspase 3 activities did not parallel the cytotoxic effects for both tumour cells, suggesting that WS-CM might induce caspase-independent non-apoptotic cell death in these transformed cell types. (17)
Acridine orange and ethidium bromide double staining	ATI-like (alveolar type I-like)	3R4F; peristaltic pump	WS, CM	Based on ATI-like cell double staining with acridine orange and ethidium bromide to distinguish between live, early and late apoptotic cells and necrotic cells, more apoptotic cells than necrotic cells were found after ATI-like cell treatment with CSE for 4 hours and more necrotic cells after a 24-hour treatment. These results indicate that CSE induced both apoptosis and necrosis in a time-and concentration-dependent manner in ATI-like cells. (24)
Caspase 3 and caspase 7 activation	ATI-like cells	3R4F; peristaltic pump	WS, CM	A caspase activity assay was used to further define the apoptosis pathway induced by CSE. The results suggested a caspase-dependent apoptosis pathway induced by CSE in ATI-like cells. (24)
Propidium iodine staining	ATI-like, ATII cells	3R4F; peristaltic pump	WS, CM	As measured by propidium iodine staining, ATI-like cells were significantly more sensitive than ATII cells after treatment with 10% CSE for 24 hours. (24)
TdT-mediated dUTP Nick-End Labelling (TUNEL)	ATI-like cells	3R4F; peristaltic pump	WS, CM	Chromatin condensation and fragmentation in ATI-like cells after treatment with CSE using TUNEL was observed. The percentage of apoptotic cells increased in a concentration and time-dependent manner. The highest induction of apoptosis (5.8%) was observed after a 24-hour treatment with CSE. A statistically significant increase in apoptotic ATI-like cells after 24 hours of exposure to all applied concentrations of CSE, and after 4 hours of treatment with 5% and 10% CSE, was found. (24)
2′,7′ dichlorofluorescein-diacetate (H2DCFDA cleaved to yield the fluorescent DCF product)	Primary human skin fibroblasts	Unknown	WS, CM	H2DCFDA assays of overall oxidative stress demonstrated an increase in ROS in cells exposed to 0.5% or 1% CSE, but not 0.25% CSE. (26)
Senescence-associated β-galactosidase (SA β-gal) staining	Primary human skin fibroblasts	Unknown	WS, CM	The number of cells positive for SA β-gal activity was increased in CSE-exposed cells. When these results were quantified, the percentage of SA β-gal positive cells/total cell number in the CSE-exposed group was found to be significantly higher than that of the CSE-free group. (26)
SOD (superoxide dismutase) and GSH-Px (glutathione peroxidase) activity	Primary human skin fibroblasts	Unknown	WS, CM	SOD and GSH-Px activities were decreased significantly in the 0.5% or 1% CSE-exposed group, whereas 0.25% CSE only resulted in a non-significant trend toward a decrease in these values. (26)

Table 3: continued

Assay	Cell type	Product (MRTP); conditions	Substance	Results (reference)
Annexin V	Human peripheral lymphocytes	Commercial cigarette (15mg tar, 1.3mg nicotine); ISO	CSC, Cambridge method	The percentages of apoptotic cells of the NS controls and the DMSO controls were 0.64%, 1.24% in the −S9 group and 0.98%, 1.33% in the +S9 group, and no significant difference was found between the NS controls and the DMSO controls ($p > 0.05$). In the CSCs −S9 group, the percentages of apoptotic cells were 3.56%, 9.68%, 19.83%, 47.66% and 40.12% at the doses of 25–125g/ml, respectively, which were all significantly higher than that of solvent control ($p < 0.05$, $p < 0.01$). In the CSCs +S9 group, the percentages of apoptotic cells in samples at the doses of 75–125g/ml were significantly higher than that of DMSO control ($p < 0.05$, $p < 0.01$), which were 4.30%, 15.96% and 45.92%, respectively. Significant difference in apoptotic cell percentages was found between the −S9 group and the +S9 group at the doses of 25–100g/ml ($p < 0.01$), and the percentages of apoptotic cells in the CSCs +S9 group were significantly lower than those in the CSCs −S9 group at the same CSCs doses. (64)

Table 4: Apoptosis studies on smokeless tobacco products

Assay	Cell type	Product (MRTP); conditions	Results (reference)
Caspase 3 activation	THP1	2S3, CAS	ST/CAS which caused marginal cell death appeared to activate caspase 3 proportionately at the doses tested. (12)
Caspase 3 activation	101A, 101B, HGEC	2S3, CAS	ST/CAS showed no or only minimal activation (~1.1 to 1.2-fold) on caspase 3 activity in all cell lines. (17)
Annexin V	101A, 101B, HGEC	2S3, CAS	The small fractions of apoptotic or necrotic cells observed in CAS controls was not further increased by ST/CAS, in agreement with its very low or minimal cytotoxicity determined by SRB assays. (17)

diacetate method, while superoxide dismutase (SOD) activity and glutathione peroxidase (GSH-Px) activity were assessed by a colourimetric method. The authors found that proliferative capacity and growth were inhibited by WS-CM exposure in a time-dependent and concentration-dependent manner, and that fibroblasts exposed to WS-CM showed significantly increased SA β-gal activity. Further, WS-CM exposure inhibited SOD and GSH-Px activities, thereby increasing ROS levels. The authors concluded that WS-CM exposure impairs fibroblast growth and proliferation and leads to features similar to those seen in senescent cells both *in vivo* and *in vitro*.

Genotoxicity

Recent studies on the genotoxicity of cigarette smoke are summarised in Table 5; studies of smokeless tobacco are summarised in Table 6. *Salmonella typhimurium* reverse mutation (Ames) assays are summarised in Table 7.

Scott *et al.* (27) examined the resolving power of *in vitro* genotoxicity assays for TPM, and recommended a uniform statistical analysis for the Ames test, the *in vitro* micronucleus test and the mouse lymphoma assay (MLA). By employing a hierarchical decision process with respect to slope, fixed effect and single dose comparisons, the authors resolved a 30% difference in TPM genotoxicity.

Several of the studies mentioned above under *Cytotoxicity* also assessed the genotoxicity of tobacco product preparations. Aufderheide *et al.* (6) exposed *S. typhimurium* tester strains to whole smoke at the ALI, and reported a dose-dependent induction of revertants. Similarly, by using the Ames test, Breheny *et al.* (28) demonstrated the mutagenicity of the gaseous tobacco smoke toxicant, ethylene oxide, both alone and in combination with whole smoke (28). Lou *et al.* (15) found that exposure to all 12 CSCs tested induced breaks in nuclear DNA, as indicated by the Comet assay; nine CSCs also induced micronucleus formation *in vitro* (15). Messner *et al.* (16) reported the degradation of nuclear DNA for HUVECs exposed to CSC (16). Combes *et al.* (18) and McAdam *et al.* (19) found reduced bacterial mutagenicity for TPMs from experimental cigarettes made with TSS or BT tobacco, that could be further reduced by the addition of a dual carbon filter. All the TPMs tested induced micronucleus formation *in vitro*, as well as mammalian cell mutation, measured with the MLA. The authors noted that, for the Ames test, pre-incubation resulted in increased sensitivity to the effects of experimental cigarettes than plate-incorporation.

While most genotoxicity studies continue to use the Ames, *in vitro* micronucleus, Comet and mouse lymphoma assays, Arimilli *et al.* (12) measured double-stranded DNA breaks by quantifying phosphorylated H2AX histone binding. Their analysis showed that CD8 cells are more vulnerable to double-stranded DNA breaks by tobacco product preparations than are CD4 cells, and that TPM and WS-CM induced more DNA damage in monocytes. The authors concluded that exposure to combustible tobacco product preparations causes a higher level of DNA damage to the leukocyte subsets than does exposure to STE. Garcia-Canton *et al.* (29) optimised the H2AX assay by high-content screening to enable aerosol exposure of human bronchial epithelial cells (29). The results of their optimisation showed that, while a positive genotoxic response was produced at all the dilutions tested, the correlation between dose and response was low.

Guo *et al.* (30) investigated the mutagenicity of 11 CSCs, ten of which were produced under ISO conditions and one under the Massachusetts Department of Public Health intensive conditions, by using microwell and soft-agar versions of the MLA (30). Exposure to all the CSCs resulted in dose-dependent increases of both cytotoxicity and mutagenicity in both versions of the MLA. However, the mutagenic potencies of the CSCs showed no correlation with the tar yield of the cigarette or the nicotine concentrations of the CSCs. The CSCs generated under ISO conditions were more mutagenic than those generated under intense conditions on a per microgramme CSC basis. The authors concluded that, while the MLA identified different genotoxic potencies among a variety of CSCs, the results from both versions of

Table 5: Genotoxicity studies on cigarette smoke

Assay	Cell type	Product (MRTP); conditions	Substance	Results (reference)
H2AX histone phosphorylation	THP1, PBMC	3R4F; ISO	TPM, Cambridge method; WS, CM	WS-CM and TPM induced double-stranded DNA damage at significantly lower nicotine units than ST or nicotine. CD8 cells were more vulnerable to DNA damage by tobacco product preparations (TPPs) than CD4 cells. TPM, WS-CM induced more DNA damage in monocytes. Treatment with the combustible TPPs caused a higher level of DNA damage to the leukocyte subsets. WS-CM was more potent relative to TPM. (12)
Comet assay	HUVEC	Unknown	CSC	CSC (50 and 100mg/ml) increased the number of comet-positive cells already 6 hours after CSC addition. (16)
Micronuclei formation	V79	3R4F, M4A, BT (MRTP); ISO	TPM, Cambridge method	At 20 hours without S9, W862 (BT Tobacco with cellulose acetate/CR20/charcoal filter) induced fewer micronuclei than W860 (untreated BT tobacco; cellulose acetate filter) and W861 (untreated BT cavity with cellulose acetate/CR20/charcoal filter). At 3 hours ± S9, W862 induced fewer micronuclei than W861. (18)
Mouse lymphoma thymidine kinase (TK) assay	L5178Y thymidine kinase (tk) +/– cells (mouse lymphoma cell line)	3R4F, M4A, BT (MRTP); ISO	TPM, Cambridge method	W863 (BT Tobacco with cellulose acetate/charcoal filter; 60mg) was less mutagenic than W861 (untreated BT cavity with cellulose acetate/CR20/charcoal filter) in both experiments with all three treatment conditions. W862 (BT Tobacco with cellulose acetate/CR20/charcoal filter) was less mutagenic than W861 in both 3 hours with S9 experiments. Neither difference was statistically significant. (18)
Micronuclei formation	V79 cell line	TSS (MRTP), SCKS; ISO	TPM, Cambridge method	When the results were expressed on a TPM or an NFDPM basis, there was a clear and statistically significant lowering of micronucleus induction from the experimental cigarette compared to the control cigarette at each concentration of particulate matter. However, when the particulate matter solution was corrected for the amount of water, nicotine and glycerol present, by calculating the NHFDPM, there was no difference between the test and control. (19)
Mouse lymphoma TK assay	L5178Y thymidine kinase (tk) +/– cells	TSS (MRTP), SCKS; ISO	TPM, Cambridge method	When the L5178Y tk +/– mutation frequency is plotted against the concentration of TPM added, under all conditions tested, the experimental cigarette induced fewer mutations than a control cigarette. However, when the results were recalculated on an NHFDPM basis and the mutation frequency was expressed allowing for the water, nicotine and glycerol content of the particulate matter, under all conditions the dose–response curves moved together and the responses for experimental and control cigarettes lay together, within the overall uncertainty of measurement of the bioassay procedure. (19)
Mouse lymphoma TK assay	L5178Y tk+/– –3.7.2C	2R4F, experimental cigarettes with AC filter; ISO, Massachusetts Department of Public Health (MDPH)	TPM, Cambridge method	The mutagenic activity per mg TPM did not appreciably change when the more intense smoking conditions were used. There was also no difference between the mainstream smoke generated from the AC filtered cigarette, as compared to that of the reference cigarette without AC filtration, regardless of the absence or presence of the metabolic activation system. (20)
H2AX histone phosphorylation	BEAS-2B	3R4F, M4A; ISO	WS, ALI	Both reference cigarettes 3R4F and M4A produced a significant increase in γH2AX frequency (above 1.5-fold increase) compared to the air-treated control in all the dilutions tested. In general, 3R4F WMCS exposure seemed to have a more potent genotoxic effect compared to M4A WMCS exposure, especially at the most concentrated dilution 1:500. (29)

Table 5: continued

Assay	Cell type	Product (MRTP); conditions	Substance	Results (reference)
Mouse lymphoma TK assay	L5178Y tk+/– –3.7.2C	2R4F, LT, LTMAS, FF, LIP (Commercial US light low ignition propensity), LVB (Commercial US light Virginia blend), LCF (Commercial US light charcoal filtered), REC, BRI, BUR, UL (Commercial US ultra light); ISO, MDPH	CSC, Cambridge method	All 11 CSCs induced dose-related cytotoxic and mutagenic effects in mouse lymphoma cells in both the microwell and soft agar versions. Among the 11 CSCs, LT, BRI and REC were the three most potent CSCs in both versions when calculated per microgramme of CSC. LT and REC remained among the top three most potent after adjusting for the nicotine content of the CSCs. BUR was the least mutagenic in both versions when calculated per microgramme of nicotine. Expressed per microgramme of CSC, LIP and FF were the least mutagenic in the microwell or soft agar version, respectively. In both versions, the mutagenic potencies spanned a 3.5 to 4.1-fold or a 12.3 to 13.1-fold range when expressed as MF per microgramme CSC or per microgramme nicotine, respectively. In addition, the mutagenic potencies of the CSCs did not show a correlation with the tar or nicotine concentrations in the cigarettes. Comparing two CSCs produced from the same commercial cigarette (US light non-menthol brand) under two sets of smoking conditions, the smoke condensate of LT smoked under ISO conditions was more cytotoxic and mutagenic than LTMAS produced under MDPH conditions in both versions of the MLA on a per microgramme CSC basis. (30)
Micronuclei formation	Chinese hamster lung cells (CHL/IU)	2R4F; ISO, HCI	WS, ALI; GVP	WS and GVP from 2R4F showed dose-related micronucleus (MN) responses under the ISO smoking regimen. The highest average percentage of the MN frequency was 4.98% and the dosage was 0.57% of cigarette smoke in WS exposure. In GVP exposure, the highest average percentage of the MN frequency was 5.42% and the dosage was 1% of cig. With the increase in the percentage of cig. values, the cytotoxicity, the decrease of viable cell count, was also observed. WS and GVP from 2R4F showed dose-related MN responses under the HCI smoking regimen. The highest average percentage of the MN frequency was 5.22% and the dosage was 0.32% of cig. in WS exposure. In GVP exposure, the highest average percentage of the MN frequency was 5.7% and the dosage was 0.62% of cig. With the increase in the percentage of cig. values, the cytotoxicity, i.e. the decrease of viable cell count, was also observed. (31)
Comet assay	A549, BEAS-2B	3R4F; ISO	WS, ALI	For all experiments and both cell lines, a clear dose-dependent increase in DNA damage was seen, demonstrating the genotoxic potential of WS. In A549 cells, the comparison between the control and all WS dilutions showed statistically significant differences with regard to DNA damage, expressed as tail intensity ($p < 0.001$). The increases in response to WS over the control varied from 5.2-fold to 17.3-fold, indicating a clear dose-response for all assays. For the BEAS-2B cell line, the increase of DNA damage in treated cells was also statistically significant when compared to control ($p < 0.001$). The manifold increases in damage in response to WS over the SA control were up to 3.9-fold, demonstrating a clear genotoxic effect. Exceptions were found for two of three experiments (same-day assay) of the highest dilution (4L/min), where no statistically significant difference was seen. (32)
Comet assay	A549, BEAS-2B	A, B, C, D (see Table 1); smoke was aspirated into a glove box chamber by a vacuum pump	CSE	Extracts from unfiltered cigarette smoke induced significant direct DNA damage, to a larger extent in A549 cells. Filtered cigarette-smoke extract induced a significant direct DNA damage at 5–10%. A significant induction of oxidative DNA damage was found at the highest CSE concentration in both cell types (by smoke extracts from B and C cigarettes in A549 cells, and from A and D cigarettes in BEAS-2B cells). Smoke extracts from filter cigarettes induced less direct DNA damage than those from unfiltered cigarettes in A549 cells, probably due to a protective effect of filter. In BEAS-2B cells the smoke extract from the B-cigarette showed the highest genotoxic effect, with a concentration-dependent trend. (33)
Cytokinesis-block micronucleus (CBMN) assay	FE1	Brand 1: flue-cured, full-flavour Brand 3: blonde, king size Brand 5: flue-cured, light, king size; ISO	CSC	The cytokinesis-block micronucleus assay in FE1 cells showed a mean spontaneous frequency of micronucleated cells of 10.1 ± 1.1 cells per 1000 scored binucleates. Exposure of FE1 cells to CSC resulted in an increase in overall MN frequency, suggesting that all brands were capable of inducing cytogenetic damage. The most potent activity was observed for Brand 3, followed by Brand 5 and Brand 1. Brand 1 elicited the weakest response, with only the highest tested concentration yielding a response significantly greater than the solvent control. (34)

Table 5: continued

Assay	Cell type	Product (MRTP); conditions	Substance	Results (reference)
LacZ transgene mutagenicity	FE1	Brand 1: flue-cured, full-flavour Brand 3: blonde, king size Brand 5: flue-cured, light, king size; ISO	CSC	The LacZ transgene mutagenicity assay in FE1 cells showed a mean spontaneous mutant frequency (± standard error) of 34.3 ± 3.8 and 22.8 ± 0.7 × 10^{-5}, without and with exogenous S9 activation, respectively. The mean response for the positive controls was 693.5 ± 36.4 and 88.3 ± 6.4 × 10^{-5} for 0.4μM BaP and 2μM PhIP, respectively. (34)
CBMN assay	Human peripheral lymphocytes	Commercial (China): filtered, flue-cured type (15mg tar, 1.3mg nicotine, 15mg CO); ISO	CSC, Cambridge method	The MN frequency of NS controls and DMSO controls were 2.67‰, 4.33‰ in the −S9 group and 4.00‰, 5.33‰ in the +S9 group, respectively, and the difference in MN frequency between NS controls and DMSO controls was not significant ($p > 0.05$). In the CSCs −S9 group, the MN frequency in samples at the doses of 25–125μg/ml were 12.00‰, 12.67‰, 14.33‰, 16.53‰ and 17.38‰, respectively, which were significantly higher than that of the DMSO control ($p < 0.01$). In the CSCs +S9 group, the MN frequencies in samples at the doses of 75–125μg/ml were significantly higher than that of the DMSO control ($p < 0.01$), which were 10.33‰, 12.00‰ and 14.13‰, respectively. Although MN frequencies in the −S9 group were higher than those in the +S9 group at the same doses, the difference in MN frequencies at the same doses between the CSCs −S9 group and the CSCs +S9 group was not significant ($p > 0.05$). (64)
Comet assay	Human peripheral lymphocytes	Commercial (China): filtered, flue-cured type (15mg tar, 1.3mg nicotine, 15mg CO); ISO	CSC, Cambridge method	The percentage tail DNA of cells exposed to 75μg/ml CSCs −S9 was 31.99%, which was significantly higher than that (14.79%) of cells exposed to 75μg/ml CSCs +S9 ($p < 0.01$). The percentages of tail DNA of cells 240 minutes after exposure in the CSCs −S9 group and the CSCs +S9 group were 6.01% and 4.92%, respectively, which did not increase significantly, as compared with DMSO controls ($p > 0.05$). DNA repair rates in both of the −S9 group and the +S9 group increased with repair time. The percentages of tail DNA of cells at 30–120 minutes repair time points in the +S9 group were significantly lower than those at corresponding repair time points in the −S9 group ($p < 0.01$). DNA repair rates at 30–120 minutes repair time points in the +S9 group were significantly higher than those at the corresponding repair time points in the −S9 group ($p < 0.05$, $p < 0.01$). (64)
Comet assay	HMy2.CIR	Cigarette 1 (3mg tar, 0.3mg nicotine, 3mg CO), Cigarette 2 (15/1.3/15); ISO	CSC, Cambridge method	In the cigarette 1 group, the range of % tail DNA was from 2.21% to 4.03%, and the % tail DNA at doses of 12 × 10^{-3}–14 × 10^{-3} cigarette/ml was significantly higher than that of DMSO control (2.09%, $p < 0.01$). In the cigarette 2 group, the range of % tail DNA of samples was from 9.35% to 71.15%, which were all significantly higher than that of the DMSO control (2.20%, $p < 0.01$). It was found that % tail DNA in the cigarette 2 group was significantly higher than that in the cigarette 1 group at each dose ($p < 0.01$). (65)
Micronuclei formation	V79	3R4F, M4A, TSS (MRTP), BT (MRTP); ISO	TPM, Cambridge method	All PMs induced dose-related increases in cytotoxicity in the three treatment conditions, and dose-related increases in the frequency of micronucleated binucleate cells in two treatment conditions (3 hours ± S9). Increases in the frequencies of MnBn cells were also observed in the 20-hour −S9 treatment with all PMs, but they were not always dose-related. The most sensitive treatment was 3 hours without S9, where up to 5-fold inductions of MnBn cells were observed. Consistent with historical data, M4A PM induced more MnBn cells than PM from 3R4F. The differences were small, but statistically significant in all three treatments. (67)
Mouse lymphoma TK assay	L5178Y (tk)+/– cells	3R4F, M4A, TSS (MRTP), BT (MRTP); ISO	TPM, Cambridge method	All PMs induced dose-related increases in cytotoxicity and MF, in all treatment conditions (3 hours ± S9 and 24 hours −S9), although some dose-responses did not exceed the Global Evaluation Factor (GEF). Consistent with historic data, M4A PM gave higher mutagenicity than 3R4F PM. This difference was statistically significant in four of the six experiments. Concentration-related increases in MF for each of the pairs of control and treatment PM samples were obtained. Viabilities for all of the concentrations of all of the PMs tested generally exceeded 70% in both experiments, irrespective of treatment parameters. When the data for the two experiments in each treatment condition were combined, TSS1 and BT1 mutagenicities were lower than that of CC1 PM, and the reductions were statistically significant. (67)

Table 5: continued

Assay	Cell type	Product (MRTP); conditions	Substance	Results (reference)
Comet assay	Human peripheral lymphocytes	Commercial (China): filtered, flue-cured type (15mg tar, 1.3mg nicotine, 15mg); ISO	CSC, Cambridge method	The % tail DNA of samples in the CSCs −S9 and +S9 groups at the doses from 25μg/ml to 125μg/ml were 20.35% to 55.47% and 11.06% to 21.58%, respectively, which were significantly higher than those of the DMSO −S9 and +S9 controls ($p < 0.01$; 7.67% and 7.13%, respectively). The Olive Tail Movements (OTMs) of samples in the CSCs −S9 and +S9 groups at doses from 25μg/ml to 125μg/ml were 5.12 to 30.75 and 1.54 to 4.52, respectively, which were significantly higher than those of the DMSO −S9 and +S9 controls ($p < 0.05$ or $p < 0.01$; 1.07 and 1.13, respectively). Moreover, there were significant differences in % tail DNA and OTMs between the CSCs −S9 and CSCs + S9 groups at all exposure doses ($p < 0.05$ or $p < 0.01$). (68)
T-cell receptor (TCR) gene mutation test	Human peripheral lymphocytes	Commercial (China): filtered, flue-cured type (15mg tar, 1.3mg nicotine, 15mg CO); ISO	CSC, Cambridge method	MFs-TCR of samples in the CSCs −S9 and +S9 groups at doses between 25μg/ml and 125μg/ml were 5.08×10^{-4}, 6.68×10^{-4}, 10.88×10^{-4}, 16.80×10^{-4} and 15.70×10^{-4}, respectively, and 3.84×10^{-4}, 6.27×10^{-4}, 7.79×10^{-4}, 12.70×10^{-4} and 11.70×10^{-4}, respectively, which were significantly higher than those for the DMSO −S9 and +S9 controls ($p < 0.01$; 2.76×10^{-4} and 2.47×10^{-4}, respectively). At CSC exposure doses of 25μg/ml, 75μg/ml, 100μg/ml and 125μg/ml, significant differences in MFs-TCR between the CSCs −S9 and CSCs + S9 groups were seen ($p < 0.01$). The mutant frequency of the TCR gene was calculated as the number of $CD3^{-}CD4^{+}$ cells divided by the number of total $CD4^{+}$ cells, and it was obvious that the number of $CD3^{-}CD4^{+}$ cells clearly increased with CSC concentrations. (68)

Table 6: Genotoxicity studies on smokeless tobacco products

Assay	Cell type	Product (MRTP); conditions	Results (reference)
H2AX histone phosphorylation	THP1, PBMC	2S3	ST/CAS appears to be ineffective in causing DNA damage at low concentrations tested in this study. (12)
Micronuclei formation	V79	2S3, water three commercial Swedish snus (SWS; MRTPs): G, water CPS, water CDM, water experimental (flavouring agent) SWS: CDM2, water, DMSO	There were several incidences of micro-nucleated binucleate cells (MNBN) that reached statistical significance when compared with the concomitant solvent control; in only two cases did the incidences exceed the historical range for the solvent control. The first of these cases was for sample CDM2, water extraction, under 3 + 17 hours, −S9 conditions. At the highest concentration tested the mean MNBN response was 4.1%, compared with 0.85% for the concomitant solvent controls and with 2.9% for historic solvent controls. However, there was considerable cytotoxicity (70%) associated with this response. The second case was the 2S3 reference product, under 3 + 17 hours, +S9 conditions. At the highest concentration tested, the mean MNBN response here was 4.08%, compared with 0.89% for the concomitant solvent controls and with 2.8% for historic solvent controls. Cytotoxicity was not excessive in this case (22%), and so the 2S3 response was unequivocally positive. (35)
Mouse lymphoma TK assay	L5178Y tk+/– –3.7.2C	2S3, water three commercial Swedish snus (SWS; MRTPs): G, water CPS, water CDM, water experimental (flavouring agent) SWS: CDM2, water, DMSO	There were several mean mutant frequencies (MFs) that reached statistical significance when compared with the solvent control; these differences were sporadic with no apparent dose–response relationship. There were two clear exceptions, both under 24-hours −S9 conditions. The first was sample CDM, where the two highest treatments both had mean MFs that were significantly greater than the mean for the solvent control. The second was sample CDM2, water extraction, where the three highest treatments all had mean MFs that were significantly greater than the mean for the solvent control. Significant linear trends in MFs were noted for several of the 3-hour +S9 and 24-hour −S9 conditions; none were observed for the 3-hour −S9 conditions. There were two cases of mean MFs greater by at least 126 revertants per million cells than the mean for the solvent control, and these were for the same samples as those noted above for conventional statistical significance. The highest treatments for the 24-hour –S9 conditions with samples CDM and CDM2, water extraction, had mean MFs of 270 and 327, respectively, compared with the mean value for the solvent control group of 103. The two positive responses described above were both associated with considerable toxicity. (35)

Table 7: *Salmonella typhimurium* reverse mutation studies on cigarette smoke and smokeless tobacco products

Strains	Product (MRTP); conditions	Substance	Results (reference)
TA98, TA100	K3R4F (9.4mg tar), K1R5F (1.67mg tar), CM5 (15mg tar), C1 (7mg tar); ISO	WS, ALI; GVP, ALI	TA98 detected mutagenicity of whole smoke of all analysed brands in a dose-dependent manner. Mutagenicity increased upon increasing the tar content of the cigarettes, except in the monitor cigarette CM5 which revealed a mutagenic activity below that of brand C1. K3R4F showed the highest mutagenicity, followed by brand C1, CM5 and K1R5F. The GVP was analysed with strain TA100. Mutagenicity increased with rising tar content of the cigarettes. The highest mutagenicity was detected with brand C1, followed by brands CM5, K3R4F, and K1R5F. (6)
TA98, TA100, TA102, TA1535, TA1537	2R4F, M4A, BT (MRTP); ISO	TPM, Cambridge method	In TA98 with S9, the reference PMs (2R4F and M4A) behaved consistently with historical data, with 2R4F being more mutagenic than M4A. W863 (80% BT tobacco, with a carbon filter) induced the lowest number of revertants with this strain in all four experiments. PMs from cigarettes with no BT tobacco (W860 and W861) exhibited the highest mutagenic potency, except for one experiment, when W864 exhibited the highest value. In pairwise statistical comparison tests, it was found that the mutagenic potencies for W862 were significantly lower ($p < 0.05$) than the corresponding values for W861 in three of four experiments. Cigarettes W861 and W862 had the same filter (CR20 and charcoal), but W862 contained 80% BT tobacco. The other consistent and statistically significant differences, observed in all four TA98 experiments, were the significantly lower mutagenic potencies ($p < 0.05$) for W863, compared with the corresponding values for W860 and W861. In the four experiments with TA100, in the presence of S9, only very slight increases in revertants were seen, mainly for reference sample 2R4F. In one experiment with TA1537 and S9, clear concentration-related increases in revertants were seen for all of the PMs, with reference sample 2R4F being the most mutagenic, followed by the other reference sample M4A, which was closely followed by the other PMs, except for W862 and W863 which were mutagenic only at the top concentration tested. (18)
TA98, TA100, TA102, TA1535,	TSS (MRTP), SCKS; ISO	TPM, Cambridge method	For TA98 and TA1537, calculated on a TPM and NFDPM basis, the revertants per microgramme obtained for experimental cigarettes were lower than those of matched control cigarettes. All differences were statistically significant except for S620, which contained 30% TSS. With the TA100 strain, the effects of experimental cigarettes were not statistically significantly lower than those of control cigarettes, except for R817; but this strain typically gave lower overall responses than TA98 and so differences were difficult to observe. Once again, when the revertants per microgramme were calculated on an NHFDPM basis, there were no statistically significant differences between any of the samples, for any responder strain. (19)
TA98, TA100, TA102, TA1535, TA1537	2R4F; Electrically Heated Cigarette Smoking System (EHCSS, MRTP): EHCSS-K6, EHCSS-K6M, EHCSS-K3, 6mg tar delivery: M6UK (UK), M6J (Japan), 1mg tar delivery: Philip Morris One (PM1), Lark One (Lark1); ISO, HPB	TPM, Cambridge method; GVP	In the most sensitive assay parameters, strains TA98, TA100, and TA1537 with S9 metabolic activation, the bacterial mutagenicity of TPM from the EHCSS series-K cigarettes was always lower than that of TPM from the CC. The bacterial mutagenicity showed a broad response range as a function of smoking intensity for all cigarettes smoked under HPB conditions. An increase in puffing intensity was generally associated with an increase in TPM and nicotine yield as well as in bacterial mutagenicity of TPM. Bacterial mutagenicity of TPM from the EHCSS-K6 generally increased with increasing puffing intensity. Considering the complete range of HPB conditions applied, 26-fold (TA98), 18-fold (TA100), and 36-fold (TA1537) increases in bacterial mutagenicity of TPM were observed with S9 metabolic activation. Similar trends were observed for the bacterial mutagenicity of TPM without S9 metabolic activation. Also for the EHCSS-K3, bacterial mutagenicity of TPM increased with increasing puffing intensity exhibiting 12-fold (TA98), 13-fold (TA100), and 15-fold (TA1537) increases. Bacterial mutagenicity of TPM from the CC in the presence of S9 metabolic activation also showed a clear increase with increasing puffing intensities and similar trends in the absence of S9 metabolic activation. (21)
TA98, YG1041, YG5161	Brand 1: flue-cured, full-flavour Brand 3: blonde, king size Brand 5: flue-cured, light, king size; ISO	CSC	All CSC samples were mutagenic in the standard frameshift test strain TA98, as well as in YG1041, which has enhanced sensitivity to nitroarenes and aromatic amines, and YG5161, which has enhanced sensitivity to unsubstituted PAHs. TA98 potency values were always lowest (0.35–0.59rev/μg TPM), followed by YG5161 (0.51–0.73rev/μg TPM), and YG1041 (0.80–1.12rev/μg TPM). For all brands investigated, mutagenic potency values obtained for YG1041 were significantly greater than those for both TA98 and YG5161 ($p < 0.0001$). An increase in mutagenic potency on YG1041, relative to TA98, indicated that aromatic amines are an important determinant of CSC mutagenic activity. In addition, an increase in mutagenic potency on YG5161, relative to TA98, indicated that unsubstituted PAHs are also determinants of CSC mutagenic activity. With respect to the brands, Brand 3 consistently yielded the highest mutagenic potency value. The results show that Brand 3 was significantly more potent than Brands 1 and 5 on TA98 ($p < 0.0001$ and $p < 0.01$), significantly more potent than Brand 1 on YG1041 ($p < 0.0001$), and significantly more potent than Brand 5 on YG5161 ($p < 0.004$). In addition, Brand 5 was significantly more potent than Brand 1 on YG1041 ($p < 0.006$). (34)

Table 7: continued

Strains	Product (MRTP); conditions	Substance	Results (reference)
TA98, TA100, TA102, TA1535, TA1537	2S3, water three commercial Swedish snus (SWS; MRTPs): G, water CPS, water CDM, water experimental (flavouring agent) SWS: CDM2, water, DMSO	Not applicable	For both conditions, there were several mean revertant counts that reached statistical significance when compared with the solvent control; these counts were often limited to the highest concentration (i.e. there was little or no evidence for a dose–response relationship). There were three cases where the effects of the two highest concentrations were both significantly greater than that of the solvent control: sample G for TA102 in the −S9 condition, sample CDM for TA1537 in the −S9 condition, and sample CDM2 in the water extraction, TA102, −S9 condition. There were only two cases of a doubling (or a tripling) of the mean value for the solvent control, but these were not the same two cases as those described above. The cases here were sample G, TA1535, −S9 (mean of 45 revertants compared to 11, a factor of 4.1; Table 2), and sample CPS, TA1535, −S9 (mean of 43 revertants compared to 11, a factor of 3.9). (35)
TA98, TA100, TA102, TA1537, TA1535	3R4F, M4A, TSS (MRTP), BT (MRTP); ISO	TPM, Cambridge method	In TA98, TA100 and TA1537, the mutagenicities of BT1 were lower than those of CC1 and TSS1 in all of the plate incorporation experiments. The reductions in mutagenicity were statistically significant in TA98 and TA1537. In strains TA98 and TA1537, the pre-incubation mutagenicities of BT1 were lower than those of CC1 and TSS1. This was also observed with TA100, but only at the non-linear part of the dose-response. (67)

the assay were comparable. Consistent with these results, Okuwa *et al.* (31) compared the mutagenicity of whole smoke, generated under either ISO or HCI conditions, to Chinese hamster lung cells at the ALI, and found that whole smoke generated under ISO conditions induced micronucleus formation *in vitro* to a greater extent than did that generated under HCI conditions (31).

Weber *et al.* (32) exposed human lung epithelial and normal bronchial cells to whole smoke at the ALI, and assessed nuclear DNA damage by using the Comet assay (32). The authors reported a reproducible dose–response relationship between DNA damage and increased whole smoke dose in both cell lines, and concluded that the combination of the Comet assay with exposure to whole smoke represents a valuable new *in vitro* test system to screen and assess DNA damage in human lung cells. Cavallo *et al.* (33) exposed these same cell types to WS-CM from commercial filter and non-filter cigarettes and used the Comet assay to measure nuclear DNA damage in A549 cells (33). The authors found that extracts from filter cigarettes induced less DNA damage than those from unfiltered cigarettes.

Yauk *et al.* (34) analysed the genotoxic effects of exposure to CSCs from full-flavour, blonde and light commercial cigarettes (34). The authors found that the exposure of FE1 cells, derived from Muta®Mouse (a transgenic mouse strain in which a bacterial *lacZ* gene is integrated) lung epithelium, resulted in an increase in overall micronucleus frequency for all three brands. In the Ames test, all the CSCs were mutagenic in the standard frame-shift strain, TA98, as well as in YG1041, which has enhanced sensitivity to nitroarenes and aromatic amines, and YG5161, which has enhanced sensitivity to unsubstituted polycyclic aromatic hydrocarbons. However, the CSCs failed to induce *lacZ* mutations in FE1 cells. Likewise, no clear brand-specific changes in gene expression induced by CSCs were observed.

Coggins *et al.* (35) assessed STEs from three commercial brands of Swedish snus (a moist powder tobacco product; SWS), an experimental SWS (with added flavouring), and reference moist snuff. The authors reported that results for SWS in the Ames and *in vitro* micronucleus tests and the MLA were broadly negative, with positive responses limited to the highest concentrations that were often associated with cytotoxicity. The authors noted that their results are consistent with the large amount of epidemiological data from Sweden, which shows that SWS is associated with considerably lower carcinogenic potential when compared with cigarettes.

Inflammation

As summarised in Table 8, Mulligan *et al.* (36) exposed primary human sinonasal epithelial cells (cultured from mucosal biopsy specimens) and NHBE cells to WS-CM, and reported both the secretion and intracellular production of the pro-inflammatory cytokine interleukin (IL)-8 in a dose-dependent manner. Furthermore, this up-regulation could be suppressed by treatment with SOD, which converts toxic superoxide into less reactive peroxides. The authors suggest that the ability to attenuate cigarette smoke-induced inflammation with SOD could provide a therapeutic/preventative approach for individuals who are exposed to cigarette smoke.

St-Laurent *et al.* (37) compared two *in vitro* models of cigarette smoke exposure by analysing the effects of WS-CM and whole smoke on the release of monocyte chemo-attractant protein (MCP-1), IL-10, and vascular endothelial growth factor (VEGF) by primary rat bronchial epithelial (NRBE) cells. MCP-1 release was increased after three days of exposure to WS-CM, but was inhibited by exposure to whole smoke at the ALI. The production of IL-10 was reduced after three days in both models, and no difference was observed in the production of VEGF. The authors concluded that WS-CM and whole smoke modulate bronchial epithelial cell inflammatory mediator production differently, and that the model of cigarette smoke exposure used can influence the data obtained.

Arimilli *et al.* (12) found that, while TPM, WS-CM and STE all induced detectable levels of IL-8 secretion, the combustible tobacco product preparations were significantly more potent than was STE (12). The authors concluded that there is greater risk from the use of combustible tobacco products than from non-combustible tobacco products, such as smokeless tobacco products.

Cell Transformation

Weisensee *et al.* (38) reported the first use of the Bhas 42 cell transformation assay with cigarette smoke, demonstrating that TPM from a reference cigarette induced a dose-dependent increase in Type III foci and a significant increase, up to 20-fold, in focus formation at moderately toxic concentrations. The authors concluded that, because this assay is fast and yields reliable results, it may be useful in product assessment, as well as for further investigation of the non-genotoxic carcinogenic activity of tobacco smoke-related test substances. In particular, the authors suggested that the assay could be used to replace the *in vivo* skin painting assay in mice.

Genomics and Transcriptomics

Gene expression studies with cigarette smoke are summarised in Table 9.

Table 8: Inflammation studies on cigarette smoke, electronic cigarette vapour, and smokeless tobacco products

Assay	Cell type	Product (MRTP); conditions	Substance	Results (reference)
IL-8 secretion	THP1, PBMC	3R4F, ISO; 2S3, CAS	TPM, Cambridge method; WS, CM	TPM and WS-CM treatments resulted in dose-dependent increases in the secretion of IL-8 in PBMCs and HL60, which decreased at higher concentrations, presumably due to exposure-related cell death. The THP1 cell line showed a moderate secretion of IL-8 with lower concentrations of WS-CM but not with TPM. Whereas nicotine and ST/CAS induced dose-dependent IL-8 secretion, the cytokine induction occurred at much higher nicotine units of exposure compared to the combustible TPPs. (12)
IL-8 secretion	HSNE, NHBE	3R4F	CSE	CSE treatment of HNSE cells increased secreted IL-8 levels significantly when compared with control ($p = 0.0119$). Furthermore, SOD treatment was able to significantly reduce CSE-induced IL-8 secretion ($p = 0.0211$). Similar to the sinonasal epithelium, the bronchial epithelial cells showed dramatic increases in IL-8 secretion in response to CSE ($p = 0.001$). Furthermore, SOD treatment was able to significantly decrease CSE-induced IL-8 secretion ($p = 0.0145$). (36)
MCP-1, IL-10 and VEGF release	NRBE (primary rat bronchial epithelial cells)	2R4F	CSE; WS, ALI	After three days, the production of MCP-1 from the air control cells (CTL) was significantly increased compared with the baseline in the CSE model. Cigarette smoke exposure by using CSE and the smoking chamber significantly decreased IL-10 production compared with air control cells. After three days of exposure to CSE or the smoking chamber, concentration of VEGF was not significantly different between exposed cells and air control cells, although the smoking chamber exposure tended to reduce VEGF production. In contrast to CSE exposure, bronchial epithelial cells exposed to cigarette smoke in the smoking chamber produced significantly less with air control cells. Cigarette smoke exposure by using CSE and the smoking chamber significantly decreased IL-10 production compared with air control cells. After three days of exposure to CSE or the smoking chamber, concentration of VEGF was not significantly different between exposed cells and air control cells, although the smoking chamber exposure tended to reduce VEGF production. (37)
Bio-Plex cytokine assay	HaCaT, A549	UK research cigarette (12mg tar, 1.1mg nicotine), e-CIG mixtures (balsamic flavours with or without nicotine, Cloudsmoke, Terna Trade; MRTP)	WS, ALI; e-cigarette vapour, ALI	Increases of cytokine/chemokine release were found in the two cell lines when PDGF-BB, basic FGF, IL-8, IL-12, IL-17, GM-CSF, IP-10, MCP-1 and MIP-1b were analysed. Increased release of IL-1ra, IL-10, G-CSF, IFN-c, RANTES, TNF-α and VEGF was found only in HaCaT. IL-6 release decreased both in HaCaT and A549 cells. The highest increase in HaCaT cells (1002.8 ± 26.6pg/ml) was that of IL-8 (6055.6 ± 807pg/ml) and IP-10 (3345 ± 106pg/ml). The highest increase in A549 cells was that of basic FGF (240.9 ± 10.6pg/ml). These results indicate that the basal components of e-CIG vapour, although non-toxic, contain some pro-inflammatory stimuli leading to changes in the secretome pattern depending on the employed cells lines. (23)

Table 9: Gene expression studies on cigarette smoke

Assay	Cell type	Product (MRTP); conditions	Substance	Results (reference)
Gene Expression Microarray MAANOVA Analysis	FE1	Brand 1: flue-cured, full-flavour Brand 3: blonde, king size Brand 5: flue-cured, light, king size; ISO	CSC	The overall number of differentially expressed genes was relatively similar among the brands. Gene ontology (GO) and pathway analysis of all the differentially expressed genes revealed significant pertubations associated with cell cycle, p53 signalling, apoptosis/programmed cell death, and steroid/cholesterol biosynthesis. The trends in gene expression profiles reflect a low concentration compensatory response involving metabolism, antioxidant defence, and growth stimulation, followed by a high concentration cytotoxic response involving p38/JNK-dependent DNA repair, cell cycle arrest, and apoptosis. (34)
GSEA (gene-set enrichment analysis)	NHBE	3R4F	WS, ALI	CS exposure induced a comparable pattern of upregulated genes in both *in vitro* and human airway epithelium, even if the exposure level was high (current smokers) or low (low CS exposure and *in vitro* single exposure). Up-regulated AIR-100 leading edge genes from *in vivo* smoking up-regulated gene signatures contained genes coding for proteins involved in: a) the metabolism of xenobiotics by cytochrome P450; b) the redox balance; c) glutathione metabolism; and d) the pentose phosphate pathway and solute transport activity. (42)
Real-time PCR arrays	NOE (primary cultured human oral epithelial cells)	Marlboro Menthol Smooth (MS), Carlton (ultra-low tar, UL), IR3, 2RF4, Eclipse	TPM, Cambridge method	Of the 84 human drug-metabolising genes included in the array, 23 were up-regulated or down-regulated by at least two-fold in NOE from one or more patients and/or by one or more TPMs. All of the tobacco smoke extracts led to enhanced levels of several genes, including *CYP1A1*, *NQO1*, *MGST1* and *HSD17B2*. *CYP1B1* was not included in the arrays supplied by the manufacturer and was assayed separately by qPCR. It, too, was induced by TPM. Although some genes were consistently induced by all of the TPMs, others were patient-specific. (43)
Springbio antibody microarray	HMy2.CIR	Cigarette 1 (3mg tar, 0.3mg nicotine, 3mg CO), Cigarette 2 (15/1.3/15); ISO	CSC, Cambridge	The expression levels of only nine proteins in cigarette 2 sample were higher than those in cigarette 1 sample, and the expression levels of only four proteins in cigarette 2 sample were lower than those in cigarette 1 sample. Among them, retinoic acid receptor and 14-3-3 sigma proteins were significantly up-regulated in the cigarette 2 group, as compared with those in the cigarette 1 group. (65)

Talikka *et al.* (39) reviewed the significance of genomics and epigenomics in lung cancer, chronic obstructive pulmonary disease and cardiovascular disease (39). The authors observed that, while clinical and epidemiological studies rarely explain the mechanisms that link biological perturbations to exposure, predictive systems biology approaches, linking global measurements from experimental models to biological networks, can assess biological perturbations before the phenotypic outcomes manifest and can help identify the mechanisms involved. Further, the authors predicted that integration of epigenetics and genomics into these approaches will lead to the identification of a full panel of biomarkers and to a more reliable depiction of DNA damage caused by cigarette smoke. The authors speculated that it might then be possible to link specific chemicals in cigarette smoke to specific diseases, leading to the possibility of focusing research and development efforts on reducing or eliminating these chemicals.

Pleasance *et al.* (40) used massively parallel sequencing technology to sequence a small-cell lung cancer cell line (NCI-H209), and identified more than 22,000 somatic substitutions, including 134 in coding exons (40). The authors also identified a tandem duplication of exons 3–8 of chromodomain helicase DNA binding protein 7 (CHD7), as well as two additional cell lines carrying PVT1 (a noncoding RNA)–CHD7 fusion genes, indicating that CHD7 may be recurrently rearranged in this disease. The authors concluded that their findings illustrate the potential for next-generation sequencing to provide unprecedented insights into mutational processes, cellular repair pathways and gene networks associated with cancer.

Gonzalez-Suarez *et al.* (41) reported a high-content screening method with NHBE cells, to investigate the impact of acrolein, formaldehyde and catechol on 13 indicators of cellular toxicity, complemented with a microarray-based whole-transcriptome analysis. While the toxic effects of the three harmful and potentially harmful constituents (HPHCs) could be observed only at the highest doses by high-content screening, whole-genome transcriptomics revealed toxicity mechanisms, including DNA damage/growth arrest, oxidative and mitochondrial stress, and apoptosis/necrosis at lower doses and earlier time points. The authors concluded that a combination of multiple toxicological endpoints with a systems-based impact assessment allows for a more robust scientific basis for the toxicological assessment of HPHCs, allowing insight into time-dependent and dose-dependent molecular perturbations of specific biological pathways.

Yauk *et al.* (34), mentioned above in relation to genotoxicity, analysed the toxicogenomic effects of exposure to three CSCs, and found that the molecular pathways affected included xenobiotic metabolism, oxidative stress, DNA damage response, cell cycle arrest and apoptosis, and inflammation (34). However, there were no clear brand-specific changes in gene expression.

Mathis *et al.* (42) investigated whether the perturbations in gene expression induced by cigarette smoke in the epithelium of smokers' airways were reproducible *in vitro* (42). AIR-100, a human organotypic bronchial epithelium-like tissue culture, was exposed to whole smoke at the ALI, and the *in vivo* smoking and smoking cessation gene signatures were compared with the *in vitro* AIR-100 expression profiles by Gene Set Enrichment Analysis. A significant enrichment of human smokers' gene signatures, derived from public transcriptomics data sets, was found in whole smoke-exposed AIR-100 tissue. Comparison of *in vitro* microRNA profiles with microRNA data from healthy smokers highlighted various microRNAs associated with inflammation, or with cell cycle processes that are known to be perturbed by cigarette smoke in lung tissue. The authors also found a dose-dependent increase in matrix metalloproteinase 1 release by AIR-100 tissue, 48 hours after whole smoke exposure, which is in agreement with the known effect of whole smoke on collagenase expression in smokers' tissues. The authors concluded that similar biological perturbations to those observed *in vivo* in smokers' airway epithelium could be induced after a single whole smoke exposure of a human organotypic bronchial epithelium-like tissue culture. They describe the emergence of this tissue culture technology as a turning point in the development of a standard experimental airway model.

Sacks *et al.* (43) compared TPMs from filtered reference, unfiltered reference, mentholated, and ultra-low tar cigarettes, as well as a potential MRTP which primarily heats tobacco, in cultures of primary human oral epithelial cells, for their abilities to affect the metabolic activation of benzo(*a*)pyrene (BaP; a chemical to which smokers are exposed) to genotoxic products, as well as the expression of drug-metabolising enzymes. Cells pre-treated with TPM concentrations of 0.2–10μg/ml generally showed increased rates of BaP metabolism, while those treated with TPM concentrations above 10μg/ml showed decreased rates. The effects of TPMs were similar when expressed on a weight basis. All the TPMs induced the phase I proteins, cytochrome P450 1A1 and 1B1, phase II proteins, NAD(P)H dehydrogenase quinone 1, and microsomal glutathione S-transferase 1, as well as hydroxysteroid (17-beta) dehydrogenase, as assessed by qRT-PCR. The authors concluded that the pattern of gene induction at physiological levels of tobacco smoke exposure favoured the activation of BaP over its detoxification.

Conclusions

This brief overview of recent publications clearly shows that the *in vitro* testing of tobacco products is

no longer "limited to a small number of cytotoxicity and genotoxicity assays". To the contrary, the field is at the leading edge of toxicological research, employing technological innovations that include ALI exposure systems, reconstituted human tissue cultures, cell transformation assays, and high-content genomic analyses. Likewise, rather than "only isolated studies" comparing one type of tobacco product to another, recent studies assess potential MRTPs, including electronic cigarettes, snus and tobacco substitutes. Some methods can be considered already fit-for-purpose; others may require additional work before regulatory application.

Challenges in implementing scientifically relevant methods in a regulatory context have been identified (44, 45). Two major obstacles include the standardisation of methods and endpoints, and the placement of these methods and endpoints into context, in order to facilitate better understanding of their regulatory utility. While we have summarised a variety of methods and endpoints that can be used to assess the toxicity of tobacco products, questions about which of the variety of cell and tissue models, culture conditions and endpoints are most relevant, remain (46). Regulators may also struggle to compare results from studies conducted according to different protocols. It is likely that the authors of some of the publications reviewed here have considered these issues in their own laboratories, and we believe that collaborative discussion among experts in the field, and outreach to regulatory stakeholders, will be needed, in order to effectively address them. The FDA has occasionally offered unique mechanisms for accepting and qualifying new tools, including toxicogenomics data and biomarkers; both of these initiatives may offer solutions to facilitate the use of data from the methods outlined here to make regulatory decisions.

Another obstacle to the regulatory use of new test methods is that it can be difficult to clearly link the results of mechanistic studies, in particular, to endpoints of regulatory interest. For some endpoints, such as eye irritation, a clear link between *in vitro* and *in vivo* methods can be established. Traditional side-by-side validation serves to provide an understanding of the relevance and reliability of new *in vitro* methods. However, the complexity of other toxicological endpoints, and the costs of validation, require new approaches (47). Modular and 'fit-for-purpose' validation concepts offer solutions to the avoidance of expensive and lengthy prospective test method validation.

Recently, the Adverse Outcome Pathway (AOP) framework has emerged as a way to correlate toxicity information from a variety of studies at all levels of biological organisation (i.e. molecular, cellular, tissue, organ and organism; 48). AOPs can be useful for a variety of hazard characterisation-related tasks, such as building linkages between mechanistic data and *in vivo* endpoints of regulatory interest, ranking or grouping toxicants with similar mechanistic effects, and supporting the development of testing strategies (49). These uses could easily be applied to tobacco product assessment. The methods described speak to several levels of biological organisation, from molecular to complex organotypic 3-D cultures. Data from *in vitro* and *in vivo* human and non-human animal studies on various products, smoke fractions and constituents, organised into a series of linear, networked AOPs, would place the *in vitro* methods discussed here into context, provide information about the relevance of particular test methods, and determine essential endpoints for a comprehensive assessment of product safety (44, 50).

An emphasis on *in vitro* and *in vivo* human studies can help to avoid concerns over inter-species extrapolation. The toxicity of tobacco smoke, constituents and particulates often depends on particle deposition and dosimetry, as well as on biotransformation, which can differ substantially among species (5, 51—53). Furthermore, combining data from human cells and tissues with existing data from human epidemiology and clinical studies, may provide insights into potential harm reduction strategies, while avoiding the extrapolation issues often associated with *in vivo* animal studies.

In general, studies that use *in vitro* methods have the ability to assess potential MRTPs more quickly and to provide more-specific, actionable and human-relevant data than do animal studies. *In vitro* models can also better reflect genetic and environmental differences within the human population (54), which can be important for tobacco addiction (55) and toxicity (56) studies. By using mechanistic endpoints and high-throughput screens, the testing and ranking of various products and constituents is easy to envision (57). Given the wealth of available *in vivo* data on many products and constituents, *in vitro* toxicological and mechanistic data on both available and novel products can provide biological support for 'reading across' to understand the toxicity of novel products, as is done in chemical risk assessment (58). Finally, the AOP approach may provide a means of placing *in vitro* test methods and mechanistic endpoints into the human *in vivo* context, and thus expedite their regulatory acceptance. Animal studies fail to offer these scientific or practical advantages.

Note Added in Proof

The authors wish to draw the readers' attention to several recent articles that discuss electronic cigarettes (59–61), an in vitro *3-D human airway model (Epi-Airway; 62), and* in vitro *systems toxicology (63).*

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